Product-to-Parent Reversion of Trenbolone: Unrecognized Risks for Endocrine Disruption

Shen Qu,[1] Edward P. Kolodziej,[2]* Sarah A. Long,[3] James B. Gloer,[3] Eric V. Patterson,[4] Jonas Baltrusaitis,[5,6] Gerrad D. Jones,[2] Peter V. Benchetler,[2] Emily A. Cole,[2] Kaitlin C. Kimbrough,[2] Matthew D. Tarnoff,[1] David M. Cwiertny[1,7]*

[1]Department of Civil and Environmental Engineering, University of Iowa, 4105 Seamans Center for the Engineering Arts and Sciences, Iowa City, IA 52242-1527, USA. [2]Department of Civil and Environmental Engineering, University of Nevada, Reno, Mail Stop 258, Reno, NV 89557, USA. [3]Department of Chemistry, University of Iowa, Iowa City, IA 52242-1527, USA. [4]Department of Chemistry, Truman State University, Kirksville, MO 63501, USA. [5]PhotoCatalytic Synthesis Group, MESA+ Institute for Nanotechnology, Faculty of Science and Technology, University of Twente, Meander 225, P.O. Box 217, 7500 AE Enschede, Netherlands. [6]Department of Occupational and Environmental Health, College of Public Health, University of Iowa, Iowa City, IA 52242, USA. [7]Department of Chemical and Biochemical Engineering, University of Iowa, 4133 Seamans Center for the Engineering Arts and Sciences, Iowa City, IA 52242-1527, USA.

*Corresponding author. E-mail: koloj@unr.edu (E.P.K.); david-cwiertny@uiowa.edu (D.M.C.)

Trenbolone acetate (TBA) is a high-value steroidal growth promoter administered to beef cattle whose metabolites are potent endocrine disrupting compounds. We performed laboratory and field phototransformation experiments to assess the fate of TBA metabolites and their photoproducts. Unexpectedly, we observed that the rapid photohydration of TBA metabolites is reversible under conditions representative of surface waters (pH 7, 25°C). This product-to-parent reversion mechanism results in unique diurnal cycling and substantial regeneration of TBA metabolites at rates that are strongly temperature- and pH-dependent. Photoproducts can also react to produce structural analogs of TBA metabolites. These reactions occur for structurally similar steroids, including human pharmaceuticals, suggesting that predictive fate models and regulatory risk assessment paradigms must account for transformation products of high-risk environmental contaminants like endocrine-disrupting steroids.

Humans discharge a multitude of bioactive organic contaminants into receiving waters that adversely impact aquatic organisms (*1–3*). Risk assessment approaches for these contaminants often are simplistic, typically assuming that if degradation occurs, the associated ecological risk greatly decreases. However, there is growing sentiment that some environmental transformation reactions result in minimal mitigation of risk, forming products that retain bioactive moieties, exhibit greater toxicity, or affect different biological endpoints (*4, 5*).

The androgenic steroid trenbolone acetate [TBA; 17β-(acetyloxy)estra-4,9,11-trien-3-one] is an anabolic growth promoter implanted in over 20 million cattle annually (*6, 7*) with annual revenue attributable to its use likely exceeding $1 billion (*8*). Given the extensive use of TBA, its dominant metabolite, 17α-trenbolone (17α-TBOH), and other known metabolites, 17β-trenbolone (17β-TBOH) and trendione (TBO), can be widespread in agricultural environments (*9, 10*). 17α-TBOH and 17β-TBOH are potent endocrine-disrupting compounds, with concentrations as low as 9-30 ng/liter causing skewed sex ratios and reduced fecundity in fish (*7, 11*). Nevertheless, observations of TBA metabolite occurrence have not yet translated to concern, as they are believed to exhibit limited persistence in receiving waters. For example, manufacturer studies used for regulatory approvals specifically point to limited ecosystem risks of TBA metabolites due to rapid photodegradation (*12*).

Accordingly, we previously identified the structure and bioactivity of TBA metabolite photoproducts, showing that 17β-TBOH undergoes rapid photohydration (*13*) to yield 12,17-dihydroxy-estra-5(10),9(11)-diene-3-one (or simply "12-hydroxy-17β-TBOH") (*14, 15*). An analogous C12-hydroxylated photoproduct was identified for TBO (*15*), while 17α-TBOH yields both major C12 ("12-hydroxy-17α-TBOH" (*15*)) and minor C5 ("5-hydroxy-17α-TBOH") hydroxylated photoproducts (supplementary materials text and figs. S1 and S2). Because these photoproduct mixtures retained bioactivity (*15*), we focused here on photoproduct stability across a range of conditions representative of light (day) and dark (night) surface waters. When photoproduct transformation was observed, we used liquid chromatography-high resolution tandem mass spectroscopy (LC-HRMS/MS) and NMR to characterize resulting product structures and the potential for persistent bioactivity in surface waters (*16*).

Simulated day-night cycling experiments conducted over 72 hours at pH 7, 25°C revealed unexpected persistence of TBA metabolites (Fig. 1A). For example, 17α-TBOH decay during irradiation was consistently followed by concentration rebound during subsequent dark periods, with equivalent rates of photodecay and dark regeneration for each diurnal cycle. Both NMR (figs. S3 to S10) and LC-HRMS/MS confirmed that 17α-TBOH was regenerated in the dark. This dynamic behavior appears linked to the thermal (i.e., non-photochemical) instability of C5 and C12 hydroxylated photoproducts, which decayed concurrently with 17α-TBOH regrowth.

A noteworthy feature of both 5- and 12-hydroxy-17α-TBOH is their allylic alcohol moiety (Fig. 1A). Steroidal allylic alcohols are prone to dehydration, but typically under more aggressive conditions (e.g., low pH, high temperature) (*17*). However, our data suggest that 5- and 12-hydroxy-17α-TBOH dehydrate under ambient conditions, with the driving force being regeneration of the conjugated trienone system. This coupled photohydration-dehydration mechanism results in net reversibility of 17α-TBOH photolysis, a product-to-parent reversion mechanism also observed for other TBA metabolites (Fig. 1B). 17β-TBOH and TBO exhibited reduced regrowth during 12 hours dark periods (~1% of initial mass) relative to 17α-TBOH (~15%), which we attribute to their photoproducts being more susceptible to concurrent phototransformation and hydroxylation rather than dehydration (supplementary materials text and fig. S11).

We also simulated TBA metabolite transport from the photic zone of a surface water into darker regions (e.g., lake hypolimnion, hyporheic zones, or benthic sediments) by photolyzing TBA metabolite solutions to >99% transformation (pH 7, 6 hours irradiation) and then storing resulting photoproduct mixtures in the dark for 5 days at 25°C. Consistent with the diurnal cycling experiments, 17α-TBOH exhibited substantial

reversion, with over 60% of its initial mass recovered after 120 hours, while 17β-TBOH and TBO yielded 10% recovery over this period (Fig. 2A).

The rates and extents of product-to-parent reversion were highly dependent on solution conditions, with some promoting rapid and near-complete TBA metabolite regeneration. For example, $88 \pm 3\%$ of the initial 17α-TBOH mass was regenerated nearly instantaneously when photoproduct mixtures were acidified to pH 2, with slightly lower recoveries ($66 \pm 5\%$) when raised to pH 12 (Fig. 2A). Reversion of 17β-TBOH and TBO was also acid catalyzed ($65 \pm 7\%$ and $32 \pm 4\%$ recovery, respectively) but their regrowth was more limited at pH 12. We believe these recoveries at pH 2 likely reflect the maximum photoproduct mass available for reversion.

Reversion of 17α-TBOH was also enhanced at pH 9 and pH 5, at least initially, relative to pH 7 (Fig. 2B). Thus, acid- and base-catalyzed reversion will likely result in higher 17α-TBOH concentrations in mildly acidic or alkaline waters. In fact, the initial rate of reversion at pH 5 is fast enough to slow 17α-TBOH phototransformation relative to the rate observed at neutral pH (supplementary materials text and fig. S12). In manufacturer regulatory studies, slower rates of 17α-TBOH phototransformation at pH 5 relative to pH 7 were attributed, we believe incorrectly, to variations in solar irradiance from cloudy weather experienced during data collection at pH 5 (*12*).

Reversion is also temperature-dependent. At pH 7, rates of 17α-TBOH regrowth increased nearly 30-fold from 5 to 35°C (Fig. 2C), a temperature range representative of seasonal variations. Faster reversion rates at 35°C ultimately allow more complete 17α-TBOH recovery in the dark (30% over 12 hours, 75% over 60 hours). Accordingly, we expect photoproduct-to-parent reversion to be most prominent in warm, sun-lit waters, whereas colder, winter-like conditions should promote photoproduct stability by slowing dehydration.

Accounting for these effects, we can now present a more complete characterization of TBA metabolite fate in surface waters. Consistent with established pathways for allylic sterols (*17*), we propose that dehydration at moderately acidic to low pH proceeds via unimolecular (E1) elimination involving formation of a resonance-stabilized carbocation (Fig. 3). Parent regeneration from this carbocation intermediate may be accompanied by concurrent transformations, including structural rearrangements and/or reaction with naturally abundant nucleophiles, to yield complex mixtures of isomers, structural analogs, and substituted derivatives of TBA metabolites with uncharacterized bioactivity. For example, while monitoring TBO photoproduct reversion at pH 5, we observed evidence of photoproduct interconversion and formation of a presumed structural analog that co-elutes with TBO (supplementary materials text and figs. S13 to S18). These phenomena were not observed at neutral or basic pH, consistent with the proposed acid-promoted carbocation intermediate. At neutral and basic pH, dehydration is more likely to proceed via enol or enolate formation in parallel to reactions yielding higher order hydroxylated products.

Beyond model laboratory systems, we have also observed reversion in more complex aquatic matrices, including Iowa River water (Fig. 4), as well as in experiments using environmentally relevant TBA metabolite concentrations (e.g., ~420 ng/liter 17β-TBOH; fig. S19). To explore reversion in agricultural receiving waters with competing attenuation pathways (e.g., sorption, biodegradation), we dosed a small collection pond on a rangeland with manure from TBA-implanted cattle (supplementary materials text). After overnight leaching increased 17α-TBOH concentrations in the pond to 6.1 ng/liter, concentrations decreased by ~50% during the day, consistent with phototransformation. At sunset, a large (31-liter) sample was collected and processed so that subsamples of this "baseline" sample could be subjected to different storage conditions (Fig. 4). Laboratory storage at 35°C significantly increased 17α-TBOH concentrations (from 7 to 20 ng/liter; Games-Howell post-hoc

test, $P = 0.003$), whereas samples stored at 1°C were statistically identical to the baseline sample (Games-Howell post-hoc test, $P = 0.65$). However, when the temperature of these 1°C samples was subsequently raised after 24 hours to 35°C, 17α-TBOH concentrations statistically increased to 15 ng/liter (Games-Howell post-hoc test, $P = 0.005$). These data are consistent with expected trends for product-to-parent reversion dynamics under actual field conditions. Incidentally, we also observed similar decay and regrowth characteristics for a major uncharacterized, nontarget compound, likely steroidal, in our GC-MS/MS chromatograms whose occurrence was highly correlated to known TBA metabolites (figs. S20 to S22).

Although growth-promoting steroids provide indisputable economic and environmental advantages (e.g., reduced land use, nutrient loads, and greenhouse gas emissions) for animal agriculture (*18*), their use should not compromise environmental health. Contrary to prevailing assumptions of limited environmental persistence, product-to-parent reversion results in enhanced persistence of TBA metabolites via a dynamic exposure regime that defies current fate models and ecotoxicology study designs. Reversion also provides a route to novel steroidal isomers, structural analogs, and derivatives, or "environmental designer steroids", with as yet uncharacterized properties and risks. Temperature- and pH-dependent reversion rates also imply substantial uncertainty for nearly all existing TBA metabolite occurrence data. Collectively, these possibilities suggest unrecognized occurrence of TBA metabolites and/or structurally similar, bioactive transformation products that may contribute to otherwise unexplained observations of endocrine disruption in agriculturally impacted receiving waters (*19*, *20*). For example, although chemical analyses often cannot identify causative agents, a recent study indicated widespread (35% of tested waters) androgenic activity in U.S. waters (*21*).

We also recently observed photoproduct-to-parent reversion for dienogest, a potent progestin used as an oral contraceptive (*22*), and for dienedione, an illicit anabolic steroid marketed as a "bodybuilding supplement" (fig. S23). Therefore, it appears that other pharmaceutical steroids, namely those with dienone and trienone moieties, also may exhibit enhanced environmental persistence and pose greater ecological risks than currently realized. In fact, although this work focused solely on phototransformation, we believe that the propensity for reversion is more generally linked to the conjugated enone moiety of TBA metabolites and these other pharmaceuticals. Ultimately, such observations of product-to-parent reversion illustrate that comprehensive assessment of environmental transformation products should be prioritized for high-risk contaminants like endocrine disrupting steroids. Further, the use of TBA or similar steroids equally prone to unique environmental transformations may need to be re-evaluated in favor of more sustainable pharmaceutical technologies designed to ensure ecosystem protection.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1243192/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S23
Tables S1 to S15
References (*23–29*)

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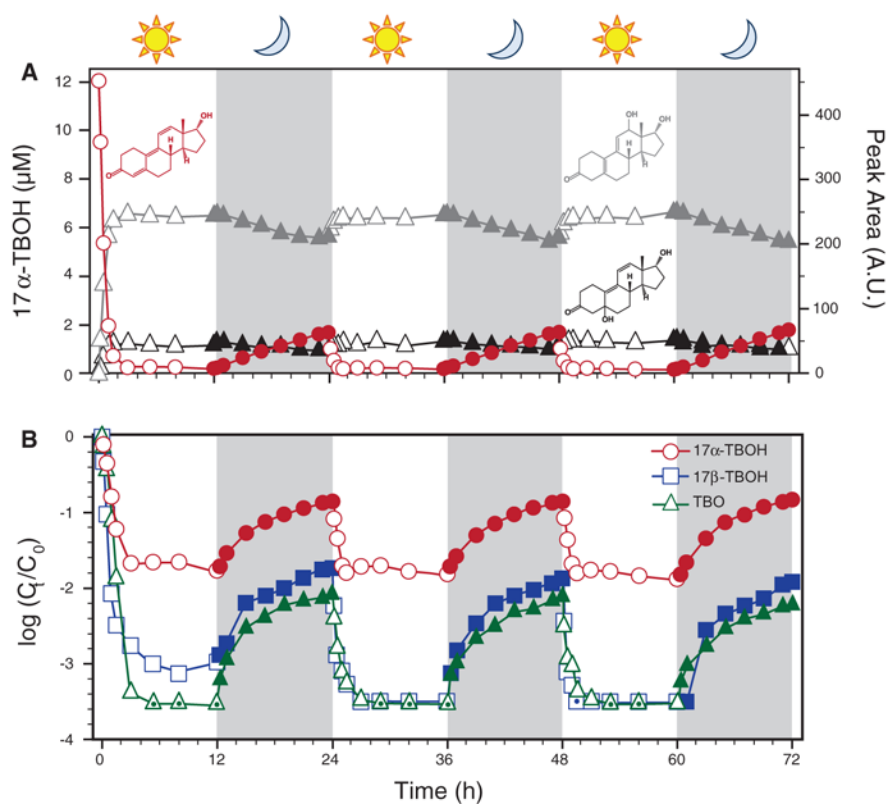Published online 26 September 2013; 10.1126/science.1243192

Fig. 1. Diurnal cycling of TBA metabolites. (**A**) Day-night cycling of 17α-TBOH (red), 12-hydroxy-17α-TBOH (gray), and 5-hydroxy-17α-TBOH (black) at pH 7, 25°C. Data for 17α-TBOH are presented as aqueous concentration (left axis), whereas peak areas from spectrophotometric absorbance (λ = 350 nm for 17α-TBOH; λ = 254 nm for photoproducts) are also presented (right axis). (**B**) Corresponding data for 17β-TBOH and TBO, where symbols with a center dot indicate values below our detection limit. In these cases, data points correspond to this limit (~3 nM) for 17β-TBOH and TBO.
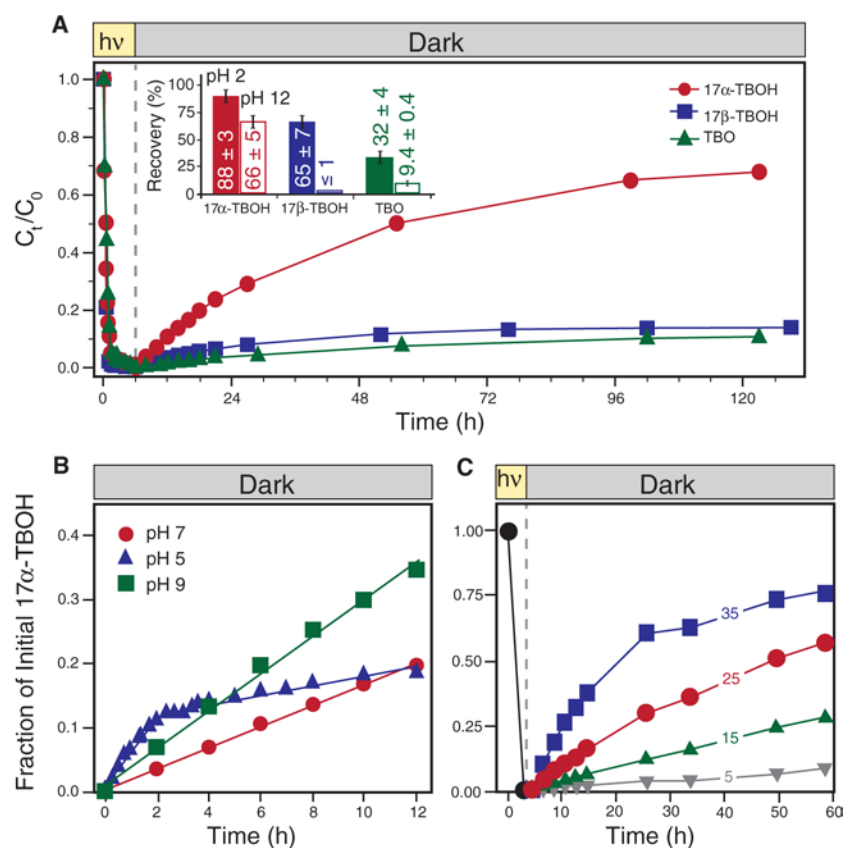
Fig. 2. Dark regrowth of TBA metabolites and the influence of pH and temperature on reversion. (**A**) Regeneration of 17α-TBOH, 17β-TBOH, and TBO in photoproduct mixtures stored in the dark (pH 7, 25°C). Inset reports the percent recovery (mean and standard deviation of triplicate analyses) when photoproduct mixtures were adjusted to pH 2 or pH 12. Also shown is the regeneration of 17α-TBOH as a function of (**B**) pH (5, 7, and 9 at 25°C) and (**C**) temperature (5, 15, 25, and 35°C at pH 7) from photoproduct mixtures. In (**B**), the initial rate of regrowth obtained from linear regression analysis was enhanced at pH 9 (0.3 µM·h^{-1}) and, at least initially (t < 2 hours), at pH 5 (0.6 µM·h^{-1}) relative to pH 7 (0.17 µM·h^{-1}). The slower rate observed over longer time scales at pH 5 (0.07 µM·h^{-1}) likely reflects differences in the dehydration of 5- and 12-hydroxy-17α-TBOH.
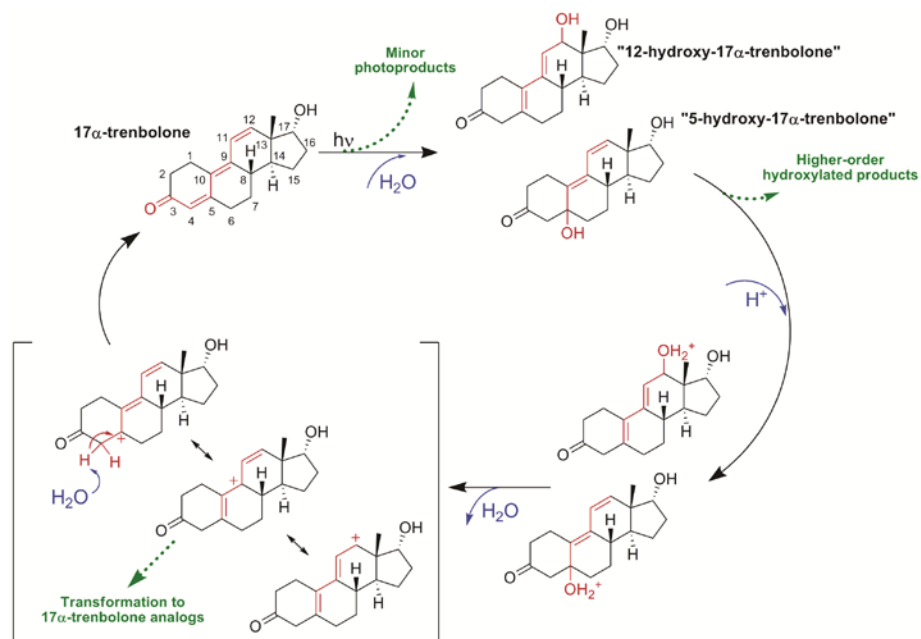
Fig. 3. Proposed acid-catalyzed reversible photohydration of 17α-TBOH. Dashed green arrows indicate pathways that break the product-to-parent reversion cycle. Red highlights detail the changes in the trienone structure during photolysis and subsequent dehydration.
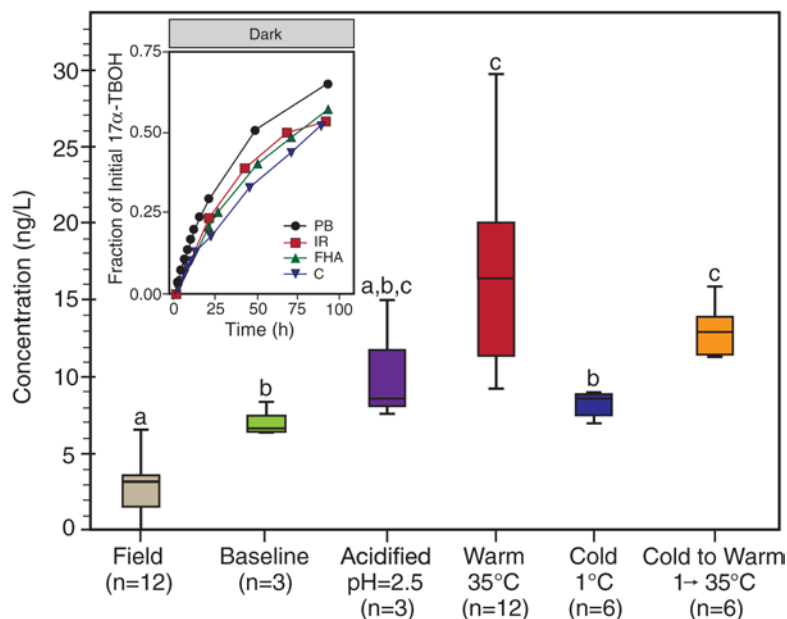
Fig. 4. 17α-TBOH dynamics in a field mesocosm and complex aquatic matrices. After dosing the collection pond (22:00), field samples ($n = 4$, in triplicate) were analyzed over 17 hours. At sunset (15:00), the "baseline" sample was processed and split into subsamples, which were either acidified to pH = 2.5, incubated ("warm", $T = 35°C$), refrigerated ("cold", $T = 1°C$), or refrigerated then incubated after 24 hours ("cold to warm", $T = 1\rightarrow35°C$). Group means are different (one-way ANOVA: $F(5,37) = 17.5$, $P <0.001$) if groups do not share the same letter as other groups (Games-Howell post-hoc test: $P < 0.05$). Lines, boxes, and whiskers represent median, inter quartiles, and minimum and maximum values, respectively. Inset shows regeneration of 17α-TBOH in photoproduct mixtures (dark, 25°C) in solutions of 10 mg/liter Fluka Humic Acid (FHA), 250 mg/ liter of bicarbonate as $CaCO_3$ (C), and Iowa River water (IR; turbidity: 15.1 NTU; alkalinity:196 mg/ liter as $CaCO_3$; total hardness: 270 mg/liter as $CaCO_3$; total dissolved organic carbon: 16.6 mg/liter; pH: 8.2.). Provided for comparison are data from a phosphate buffer (PB) system. Unless noted, all systems were at pH 7. Rates of reversion are less in Iowa River water than would be expected for its alkaline pH, likely from the slight inhibition observed in model systems with Fluka Humic Acid and bicarbonate.